If the facts don't fit the theory, change the facts.

Albert Einstein.

Abstract
1 ABSTRACT

Statins specifically inhibit HMG-CoA reductase, the enzyme that catalyzes the conversion of HMG-CoA to mevalonate, which is an early rate-limiting step in cholesterol biosynthesis in the body. These agents are highly effective in reducing total cholesterol and the low-density lipoprotein levels in several forms of hypercholesterolemia. Despite the fact that HMG-CoA inhibitors seem to be structurally similar, analytical methods for their quantitative determination in blood have been developed individually for each drug because of their different solubility, stability and optic characteristics. Atorvastatin is an HMG-CoA reductase inhibitor, which has a widespread use in the prevention of cardiovascular events. Atorvastatin is administered as the calcium salt of its active acid form, and is metabolised by CYP3A4 to two hydroxylated metabolites, o-hydroxyatorvastatin and p-hydroxyatorvastatin. About 70% of the total plasma HMG CoA reductase inhibitory activity is accounted for by active metabolites. In several clinical studies regarding statins in general, including atorvastatin, determination of the statins has been performed using alternative methods, such as enzyme inhibition assays, other than determination of the actual plasma concentrations of the compounds. Such indirect measurements are relevant when determining the HMG-CoA reductase inhibitory activity of the statin in plasma, but they do not give any further information on metabolites. Information about the actual plasma concentration of both parent compound and metabolites is of interest in phenotyping and pharmacokinetic studies. However there is no suitable method available for determination of atorvastatin and its metabolites in plasma thus aims of our study was to develop and validate a method for the detection of atorvastatin and its metabolites in plasma.
The Alliance HT HPLC with integrated system of quaternary pump and Mass spectrometry a Quattro LC tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an ESI source and Mass Lynx version 4 software were used with an Cyno 125 X 4 mm, 5µm column. Mobile phase was Acetonitrile-methanol-0.1% formic acid in water (50-30-20, v/v) and the flow rate was set to 0.5 ml/min auto sampler tray temperature was set to 10 °C and column oven temperature at 45 °C.

At the above conditions the total time of analysis in the chromatographic system was 3.5 min. and the retention times of atorvastatin, metabolites and internal standard were at around 2.4 min. Preparation of samples from healthy volunteers and plasma samples spiked with aliquots of the analytes was performed by SPE. The plasma samples (0.5 ml) were spiked with 5µg of the internal standard pravastatin and 0.5 ml of 2% formic acid in water and were vortexed for 30 seconds. The content was subsequently transferred to 1 ml C18 (100 mg) SPE cartridges (Oasis, Waters Corporations, Milford, Mass, USA) pre-conditioned with 2 ml methanol followed by 1 ml water. The cartridges were washed with 2 ml 2% formic acid in water, and the analytes were eluted with 1 ml 0.1 % formic acid in methanol after the extraction of samples, the extracts were concentrated and evaporated to dryness under a stream of N2 at around 60° C and the residues were reconstituted in 200 µl mobile phase resulting solution was vortexed for 30 seconds and than transferred to HPLC vials and than injected 25 µl in the HPLC system.

Validation of the method showed linearity within the concentration range 0.2–40 ng/ml for atorvastatin acid and 0.25–50 ng/ml for p-hydroxyatorvastatin acid and o-hydroxyatorvastatin acid (r2>0.99, n=3 for all analytes). Relative standard deviations (RSDs) of the estimated slopes were less than 15% over the whole concentration range. The intercepts of the calibration curves were shown to be not statistically
different from zero for any of the analytes. LOD was 0.06 ng/ml for atorvastatin and p-hydroxyatorvastatin, and 0.15 ng/ml for o-hydroxyatorvastatin. Recoveries for all analytes ranged between 50 and 68% on average. The specificity of the method was tested using extracted drug-free plasma from six individuals. No interfering peaks were found. Also, no interfering ion suppression or matrix effect on signal enhancement was observed. No significant concentration changes in any of the analytes were observed upon auto sampler storage at 10°C for a period of up to 24 h. Intra and Inter-day precision and accuracy for the study were performed and were found within limit. We successfully developed and validated an LC-ESI-MS-MS method coupled with SPE for the quantitative determination of cholesterol-lowering atorvastatin drug in plasma samples.

To exert an optimal pharmacotherapeutic action, an active substance should be delivered at the site of its action in an effective concentration during the desired period. To allow prediction of the therapeutic effect, the performance of the pharmaceutical form containing the active substance should be reproducible. Several therapeutic misadventures in the past (digoxin, phenytoin, primidone) testify the necessity of this reproducibility as a quality requirement. Thus the bioavailability of an active substance from a pharmaceutical product should be known and be reproducible. This is especially the case if one product is substituted for another. In that case the product should show the same therapeutic effect in the clinical situation. It is generally cumbersome to assess this by clinical studies. Assuming that in the same subject an essentially similar plasma concentration time course will result in essentially similar concentrations at the site of action and thus in an essentially similar effect, pharmacokinetic data instead of therapeutic results may be used to establish equivalence: bioequivalence.

Cardiovascular disease is the major reason of death in the Western world, and atherosclerosis is the major cause of coronary heart disease (CHD) and stroke. Hypercholesterolemia is a crucial factor in the
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development of atherosclerosis in general and CHD in particular. Treatment of hypercholestrolemia is life-long, and the drugs will be used in patients who will never encounter a CHD endpoint in spite of an increased statistical risk. Therefore, useful drugs must be effective, safe and well tolerated.

Atorvastatin, which belongs to the second generation of statins, is a synthetic reversible inhibitor of microsomal enzyme HMG-CoA reductase. It is seen that there is excessive variability in bioavailability of atorvastatin from subject to subject and moreover it is used on a long-term basis and is metabolized to active metabolites ortho and para hydroxyatrovastatin, Thus the a multiple-dose experimental model that more closely represents therapeutic use was used consequently comparative bioavailability assessments was based on steady-state plasma concentrations.

We performed the bioequivalence study of two atorvastatin formulations i.e. Test and Reference by steady state model and measured bioequivalence with both of its metabolites. This study was conducted at Synchron Research Pvt. Ltd, Ahmedabad, according to the principles of the Declaration of Helsinki and according to ICH, GCP. Institutional ethics committee approved the protocol, and the participants gave written informed consent before participation in the study. In total, 44 healthy volunteers were enrolled in this study. All participants were in good health according to medical history and physical examination. Participants had no clinically significant disease or other medical disorder that would interfere with participation in the study. The concomitant drugs, which interfere with study results, were not permitted during this study. Subjects were asked not to change their eating habits during the course of the study.

The study design was a multiple-dose, 2-treatment, 2-period, 2-sequence crossover with a study duration of 14 continuous days and a washout period of at least 28 days Participants were randomized to one
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of the two treatment groups: A and B. An oral dose of one tablet of atorvastatin 40 mg tablets (test (A) or reference (B)) was administered as per the randomization schedule every morning for 14 days for both periods. Fourteen days were enough to reach the steady state concentration. Subjects received the alternate treatment in the subsequent period following crossover with the following possible treatment sequence (B-A or A-B). The subjects visited Synchron facility everyday for dosing, at specified time from day 01 to day 13 and from day 43 to day 55. They were educated, requested and expected to maintain the fasting condition during non-in-house phase for at least 10 hours before every dose administration and 4 hour after dose in both periods. On days 01 and 43 the subjects remained in facility four hours after dose for vital monitoring after two hours of dosing. They were provided with standard meals four hours post dose. On day 13th and 55th the subjects were admitted in the Synchron facility in respective periods. On day 14th and day 56th the dosing was done as per everyday schedule after ensuring the 10-hour pre dose and 4 hour post dose fasting. The meals were provided at approximately 4 hours and 12 hours post dose, and snacks at approximately 8 hours post dose of day 14 and day 56. Water was restricted for one hour pre-dose to two hours post-dose; at other times drinking water was permitted ad libitum. Blood samples (7 ml each) were drawn in vacutainers containing K$_3$EDTA during each period at the time of the study beginning date i.e day 1 (predose), and at day 7 and 16 blood sample collection for laboratory evaluation for safety assessment. On days 11, 12 and 13 blood samples were withdrawn at 07.00 h (predose) before dosing trough concentration) for the last 3 days for both treatments A and B. On the final day (fourteenth day), a blood sample (7 ml each) was collected from subjects of treatment A at 07.00 h (predose), and then 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 16, 24, 48 hours after dosing. Blood samples (7 ml each) from subjects of treatment B were collected on the sixth day at 07.00 h (predose), and then 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 16, 24, 48
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hours after dosing. Plasma was separated by centrifugation within 1 h of collection. The separated plasma samples were then transferred to deep freezer in pre-labeled tubes below -80°C for storage. Plasma samples were analyzed for the parent compound atorvastatin, its ortho- and para-hydroxy-metabolites, by a high-performance liquid chromatography tandem mass spectrometry (LC-MS-MS) assay.

The mean, SD, and CV values were calculated for each plasma concentration of atorvastatin, ortho hydroxyatrovastatin and parahydroxyatrovastatin. Data from different sampling times for the 2 periods after drug intake were used to obtain the following parameters: The area under the plasma concentration curves at steady state (AUCss); the percent peak-trough fluctuation of plasma concentration (%PTF); the maximum concentration steady state (Cmax, ss); the minimum plasma concentration at steady state (Cmin, ss); the time to maximum concentration (Tmax, ss) after steady state, and the relative bioavailability. All pharmacokinetic variables were calculated by noncompartmental methods. Cmax, ss and Cmin, ss were read directly from the data, while Tmax, ss was determined at the respective blood-sampling times corresponding to Cmax, ss. AUCss was calculated according to the linear trapezoidal rule.

The extent of bioavailability was estimated by calculating the steady state Area under concentration time curve (AUCss). The rate of bioavailability was estimated from Cssmax and Tssmax. The relative bioavailability of the two formulations was determined from the above parameters and was considered bio-equivalent if arrived within 90% confidence interval. Relative bioavailability was expressed as a ratio.

From 41 subjects who completed the study all were male with mean age of 34 ± 10 years and the age range was 24 to 38 years. Both the preparations were generally well tolerated without any sign of clinically significant adverse effects in laboratory parameters or physical findings. No serious adverse events were registered during the course of the trial.
To test for the attainment of steady state, day-to-day fluctuations for predose concentrations (Css min) were determined. During days 11,12 and 13 (representing the first crossover, ie, dosing period 1) and days 53,54, and 55 (representing the second crossover period, ie, dosing period 2), the %fluctuation, for the test product and reference product confirmed that the steady state was achieved.

Relative Bioavailability of Atorvastatin for Test vs. Reference, the geometric mean ratios indicate that the relative bioavailability of Test compared with Reference is 98.9%, 79.1% and 84.7% for Css min, Cssmax, and AUCss respectively. The ANOVA Table for Css min, Cssmax, and AUCss of Test vs Reference showed that forCss min, there was no significant difference among formulations since, p>0.05 but for Cssmax and AUCss there was significant difference among formulations since, p<0.05.

Relative Bioavailability of Ortho-atorvastatin for Test vs. Reference, the geometric mean ratios indicate that the relative bioavailability of Test compared with Reference is 96.5%, 77.9% and 86.0% for Css min, Cssmax, and AUCss respectively. Similar to atorvastatin The ANOVA Table for Css min, Cssmax, and AUCss of Test vs Reference showed that for Css min, there was no significant difference among formulations since, p>0.05 but for Cssmax and AUCss there was significant difference among formulations since, p<0.05.

Relative Bioavailability of Para-atorvastatin for Test vs. Reference, the geometric mean ratios indicate that the relative bioavailability of Test compared with Reference is 88.6%, 82.1% and 89.6% for Css min,Cssmax, and AUCss respectively. ANOVA for AUCss, there is no significant difference among formulations since, p>0.05 but for Css min and Cssmax there is significant difference among formulations since, p<0.05.

With dose of 40mg atorvastatin the Cssmax was 15.167±6.87 ng/mL for test formulation and 20.223±12.62 ng/mL for the reference
formulation and the minimum value was as low as 5.679 ng/mL in test and 7.404 ng/mL reference. C_{\text{ss max}} for ortho-hydroxyatorvastatin was 10.17±4.64 ng/mL for test formulation and 13.769±8.18 ng/mL for the reference formulation and the minimum value was as low as 3.236 ng/mL in test and 3.941 ng/mL reference and C_{\text{ss max}} for para hydroxy atorvastatin was 3.575±1.93 ng/mL for test formulation and 5.176±6.56 ng/mL for the reference formulation and the minimum value was as low as 1.118 ng/mL in test and 1.128 ng/mL in reference.

The results of this study showed that the protocol design allowed characterization of the pharmacokinetics and bioequivalence of atorvastatin and its active metabolite orthohydroxy atorvastatin and para hydroxy atorvastatin at steady state and the multiple dosing during the first 14 days of the pretreatment period, including the administration of 14 atorvastatin tablets of 40 mg each, was sufficient to attain a steady-state.

As far as bioequivalence is concerned we can conclude that the test formulation was not bioequivalent for atorvastatin and orthohydroxy-atorvastatin to reference formulation with respect to all the pharmacokinetic [C_{\text{ss min}}, C_{\text{ss max}} and AUC_{\text{ss}}] parameters. For all pharmacokinetic parameters it did not conclude equivalence and hypothesis is accepted at the 5% level of significance i.e. 90% confidence interval for μA/μB does not lies within (0.8, 1.25).

However the test formulation was also not bioequivalent for parahydroxy-atorvastatin to reference formulation with respect to C_{\text{ss min}} and C_{\text{ss max}} and was bioequivalent with respect to AUC_{\text{ss}}. For C_{\text{ss min}} and C_{\text{ss max}} pharmacokinetic parameters it did not conclude equivalence and hypothesis is accepted at the 5% level of significance i.e. 90% confidence interval for μA/μB does not lies within (0.8, 1.25), but for AUC_{\text{ss}} it conclude equivalence.
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Genetic polymorphism in CYP3A4 is observed and expressed in about 30% of Caucasians and 60% of African Americans, which may contribute to the interindividual variability in biotransformation of CYP3A4 substrates. A prior phenotyping-genotyping screening of volunteers could identify poor metabolisers (PMs) who should not be enrolled in the trial.

Variability in drug effects has been investigated since the 1950s. The first observations were during the Second World War when it was noticed that it was mostly black American soldiers who suffered from hemolysis when using the antimalarial drug primaquine. This was later explained as glucose 6-phosphate dehydrogenase deficiency. When isoniazid was introduced as treatment for tuberculosis, it was observed that individuals either had slow or rapid acetylation of the drug.

Cytochromes P450 (CYP) is an enzyme, mainly catalyzing the oxidation of xenobiotics, in order to facilitate their excretion from the body. CYP3A4 is a polymorphic enzyme, which is responsible for the metabolism of about more than 50% of all known drugs, e.g., antihypertensives, hypcholesterolemic, etc., which show a considerable interindividual variation in their metabolic clearance. Statins have been accepted sensitive probe drug for CYP3A4 activity by the U.S.Food and Drug Administration. There are data suggesting that other metabolic pathways in addition to CYP3A proportionally metabolize simvastatin. Approximately 20% of the metabolism of simvastatin can be attributed to the polymorphic enzyme CYP2C8. Therefore simvastatin may not be considered as an ideal CYP3A probe. Atorvastatin was considered as a suitable probe drug for this study because its mainly metabolized by CYP3A4 only and it also fulfilled major requirement of probe validation proposed by Watkins and modified by Zaiglerm. Moreover it is well-tolerated, free from radioactivity and phenotyping is technically easy.
Thus the present investigation was undertaken to study the genetic polymorphism of Cytochrome P450 enzyme in Gujarat population using atorvastatin as a novel probe. A total of 125 subjects were enrolled for the study, of which 97 were men and 28 were women. The mean age of all the subjects was $26.15 \pm 4.2$ years and ranged between 20- to 35-year. For phenotyping, each healthy gujarati volunteer was given 20 mg Atorvastatin (Zivast. FDC.India), orally with 250 ml water after an overnight fasting. No food was allowed throughout the study. Subjects were not taking any concurrent medication and were asked to abstain from drinking tea or coffee 10 h before drug administration and 3 h afterwards. The post-dose samples were collected at 2 hour after drug administration. Seven millilitres of venous blood was drawn into a pre-labeled vacutainers containing K3EDTA. The samples collected were centrifuged to separate plasma, immediately within 30 minutes after receiving the blood samples from all the subjects. The separated plasma samples were then transferred to deep freezer in pre-labeled tubes below -80°C for storage. Plasma samples were analyzed for the parent compound atorvastatin, its ortho hydroxy-metabolites, by newly developed high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) method. A probit plot and a frequency distribution histogram between log MR on the abscissa and the number of the subjects on the ordinate expressed analysis of interindividual variations in the metabolism of atorvastatin. The Kolmogorov-Smirnov test was also used to test the normality of the metabolic distribution. Multiple regression was used to calculate the inflection point in the probit plot. The data of Gujarat (West Indian) subjects shows a bimodal distribution with respect to metabolism of atorvastatin. Interindividual differences of drug metabolism expressed as log MR were plotted in the form of a frequency distribution histogram with the log MR of atorvastatin hydroxylation on the x-axis and the number of subjects on the y-axis.
It may be inferred that there were 3 poor metabolizers and 122 extensive metabolizers of atorvastatin. Probit plots further confirmed bimodal distribution of Gujarat (West Indian) subjects with respect to hydroxylation of atorvastatin. The inflection point in probit plot showed bimodal distribution of the human subjects. From the inflection point in the probit plot it can be inferred that 3 of 125 subjects are poor metabolizers. Antimode value from probit plot were computed with the multiple regression method and was found to be 1.7 for log DM. Individuals demonstrating log DM values between 0.4 to 1.7 were therefore categorized as extensive metabolizers, whereas individuals demonstrating values higher than 1.7 were categorized as poor metabolizers. Of the 28 women phenotyped, one women subject was poor metabolizer. Thus the frequencies of occurrence of extensive and poor metabolizers of atorvastatin in women were 96.43% and 3.57%, respectively. Of the 97 male volunteers, two were poor metabolizers of atorvastatin, demonstrating that the frequency of occurrence of extensive metabolizers and poor metabolizers of atorvastatin in men was 97.94% and 2.06%, respectively. These observations showed that frequency of occurrence of poor metabolizer phenotype is 2.4 % in the Gujarat (West Indian) subjects. The observed frequency in Gujaratis is within the range demonstrated for other ethnic groups. For example, this polymorphism occurs with a frequency of less than 1% in Orientals, 5% in Caucasians.

It has been reported that CYP expression is altered in disease and pathophysiological states that arise from acute and chronic disease can result in CYP modifying levels of various cytokines CYP reaction products, or metabolites, have been detected in cardiovascular tissue. Various studies have documented the role of these endogenous CYP metabolites in the maintenance of cardiovascular health. Animal studies also suggest a role for CYP3A in hypertension and its observed that renal CYP3A activity is markedly increased in SHR compared with
Wistar-Kyoto (WKY) rats. Thus we tried to study the metabolism of atorvastatin a CYP3A4 probe in patients of coronary artery disease.

Patients of either sex between 18 and 75 years of age with documented Coronary artery Disease single vessel, double vessel or multiple vessel coronary artery disease confirmed with prior angioplasty were recruited from Sterling hospital Ahmedabad. The patients were excluded if were on concurrent medicines which were CYP3A4 inhibitors or had high caffeine or tobacco consumption. For phenotyping, each CAD patient was given 20 mg atorvastatin (Zivast, FDC, India) orally with 250 ml water at bedtime (nearly 8.00P.M) and the post-dose samples were collected at 2 hour after drug administration (10.00 PM). The collected samples were centrifuged to separate plasma and were analysed for the parent compound atorvastatin, its ortho hydroxy-metabolite, by a high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) assay.

The drug to metabolite ratio was plotted against the frequency and The Kolmogorov–Smirnov test was used to test the normality of the metabolic distribution in the diseased population. It is seen that the distribution was not normal as D value was 0.331, p-value< 0.0001 at alpha 0.05.

In our study the patients of CAD who are on chronic treatment with atorvastatin had an increase in the metabolic ratio of atorvastatin, as compared to normal patients there was a significant increase in the drug metabolite ratio, which was representative to the decrease in the CYP3A4 activity in CAD patients.

It was also observed that the increase in the drug metabolite ratio was proportional to the number of coronary arteries blocked, which suggest that, as the hypercholestrolemia blocks the artery there is a
proportional decrease in the CYP3A4 activity. It's reported that atorvastatin treatment increased the eNOS production. Probably the increased NOS increased the NO production that further inhibited the CYP and thus produced the increased drug metabolite ratio in patients suffering from CAD.

In conclusion the method developed by us was suitable for determination of Serum concentrations of atorvastatin and its two metabolites by a liquid chromatography-tandem mass spectrometry method LC-MS/MS. The assay showed a lower limit of quantification of 0.2ng/L for all analytes. The calibration curve was linear from 0.2–40 ng/ml for atorvastatin and 0.25–50 ng/ml for p-hydroxyatorvastatin acid and o-hydroxyatorvastatin acid and the method demonstrated good precision and accuracy.

The genetic polymorphism of human Cytochrome P450 is an important factor for the bioavailability and bioequivalence studies. A prior phenotyping of the study subjects is critical and very important for the bioequivalence studies.

The observed frequency in Gujaratis is within the range demonstrated for other ethnic groups. Gujarati population is ethinically quite close to Caucasians.

Atorvastatin was found to be effective in treatment of CAD and the patients chronically on atorvastatin showed increased metabolic ratio. There was a very good correlation between the metabolic ratio in single vessel, double vessel and multiple vessel disease patients when compared against either hypertension or diabetes, which proves that diabetes and hypertension are one of the risk factors for Coronary artery disease.